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TITLE OF THE INVENTION (280 characters max)

Immobilizing and Processing Specimens on Matrix Materials For The Identification of Nucleic Acid Sequences

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted, *Marilyn J. Stapleton*

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Date 4/14/97

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REGISTRATION NO. *(if appropriate)*

☐ Additional inventors are being named on separately numbered sheets attached hereto

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PATENT

IN THE UNITED STAES PATENT AND TRADEMARK OFFICE

IMMOBILIZING AND PROCESSING SPECIMENS ON MATRIX MATERIALS FOR THE IDENTIFICATION OF NUCLEIC ACID SEQUENCES

Inventors: Marilyn J. Stapleton and Rebecca Sundseth,

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IMMOBILIZING AND PROCESSING SPECIMENS ON MATRIX MATERIALS FOR THE IDENTIFICATION OF NUCLEIC ACID SEQUENCES

BACKGROUND OF THE INVENTION

This application is a continuation-in-part application of U. S. Patent Application Serial No. 935,637, now U. S. Patent No. 5,382,511 and U. S. Patent Application Serial No. 836,348, now U. S. Patent No. 5,451,500.

FIELD OF THE INVENTION

The present invention relates to a process and device for analyzing a biological specimen for the presence of a nucleic acid sequence for diagnostic purposes. More specifically, it relates to immobilizing small amounts of cellular or tissue specimens for the analysis of nucleic acids without extracting them from the specimen. Immobilizing cells and tissue simplifies sample preparation for a variety of different nucleic acid detection methods. In some instances the number of copies of the nucleic acid sequence of interest present in the specimen is sufficient for detection, particularly with newer, more sensitive detection methods. In other instances, the nucleic acid sequence of interest is extensive enough that detection is possible by increased labeling of the target or probe molecules. In other instances when the nucleic acid sequence of interest is rare in the specimen or when distinguishing fine sequence detail, a means to amplify the nucleic acid sequence

above background may be desirable for detection. The process and device of the invention utilizes immobilization of the biological specimen to a matrix for the purpose of detecting specific nucleic acid sequences in the specimen. Detection occurs when either a reagent that recognizes the target nucleic acid binds to targets within the biological specimen and the reagent's presence is detected in the matrix, or a first reagent modifies a specific target sequence of the specimen in a way that the modified product sequence is detected by the location where it binds to a second reagent. Means to modify a target sequence include enzymatic means to synthesize a nucleic acid sequence from one or more specific primers or ligate two sequences together based on their binding adjacently on a specific template. Means to detect the modified target sequences include binding to a second reagent that recognizes the modified product and is in a specific location, such as a probe array. Immobilized Sample Amplification, or ISA, increases the labeled molecules or number of copies of the molecular target adequately enough for detection by appropriate means. Appropriate means include, but are not limited to, detection of signals that are fluoresecent, luminescent, colorimetric, electrical, chemical or image analysis and any combinations thereof.

DESCRIPTION OF RELATED DISCLOSURES

In U. S. Patent Number 5,382,511, a process to amplify nucleic acid targets was invented wherein a biological specimen was immobilized on the surface of a microscope slide or embedded in a gel matrix, for preparing and directly amplifying the nucleic acids in

the sample. Prior to said method invention, nucleic acid amplification had been limited to solution reactions wherein the nucleic acids were first extracted from cells or tissue. A number of examples for using *in situ* amplification are given in U. S. Patent Number 5,188,963. A photomicrograph of cells which had amplified and labeled DNA was included in U. S. Patent Number 5,451,500 to show that the amplified fragments are retained in individual cells and such cells can be enumerated under microscopic observation. A number of subsequent publications support this method and even the localization of nucleic acid amplification products on cells treated in a manner in which only the chromosomes remain immobilized on the glass slides.

In some nucleic acid diagnostic tests, microscopic observation of the biological specimen to look for morphological changes is productive and informative to a diagnosis. The degree of cell fixation needed to maintain cell morphology may be counterproductive to efficient detection or amplification because the most common cell fixatives cross-link the proteins and nucleic acids. Protease digestion is frequently used to reverse crosslinking because crosslinks interfere with hybridization to target sequences or terminates DNA primer extensions during DNA synthesis, thereby reducing the efficiency of amplification or hybridization detection.

In many DNA-based diagnostic tests, maintaining cellular morphology may be irrelevant to a diagnosis as long as the origin of the specimen is known. For example, the specimen may be aspirated from a series of a patient's lymph nodes, so that a pattern of metastasis from a primary tumor may be identified by the presence or absence of specific nucleic acid tumor markers in each of the lymph nodes.

The standard research protocol for sample preparation is to lyse cells to release the nucleic acids. Nucleic acids are then separated from the cell's peptides and lipids by phenol:chloroform extraction and then ethanol-precipitated to concentrate them. Varying amounts of the sample's nucleic acids are lost during the many extraction steps. Special DNA purification materials are available, but require filtration, centrifugation, or electrophoresis and add cost to preparation.

The conventional approach for clinical specimens is to perturb the cells or viral particles from a few cells or a small amount of tissue in order to release nucleic acids into solution and use a portion of the crude extraction. When this approach fails, the nucleic acid component of the crude extract requires further purification. A variety of methods exist to enzymatically either label or amplify specific nucleic acid sequences in order that they may be detected. Amplifying DNA or RNA from clinical specimens has been difficult to optimize because the clinical specimens present so many different parameters and potential inhibitors. Quick lysis techniques give rise to unpredictable or anemic PCR reactions. Anemic reactions are a continual challenge in making a robust diagnostic test because detection depends upon reproducibly amplifying the specific targets from a complex genetic background.

The *in situ* amplification process described in U.S. Patent Numbers 5,382,511 and 5,451,500 uses enzymes such as polymerase or ligase, separately or in combination, to repeatedly generate more copies of a target nucleic acid sequence by primer extensions to incorporate new nucleotides or by ligations of adjacent complementary oligonucleotides, wherein each template generates more copies and the copies may themselves become template. By melting complementary strands of nucleic acids, the original strand and each

new strand synthesized are potential templates for repeated primer annealing or ligation reactions to make and expand the number of specific, amplified products. A thermostable polymerase with reverse transcriptase activity and a thermostable ligase are now both commercially available and increase the choice of enzymes for both RNA and DNA detections. The amplification can either be primer extensions in one direction for linear amplification, or in opposing directions, for geometric amplification. The label can either be incorporated as labeled nucleotides or labeled primers for one-step detection or labeled probes may be added whereby the probes hybridize to the amplified products for detection. Other schemes for modifying, labeling or amplifying, include, but are not limited to, PCR (Roche Molecular Systems, Branchburg, New Jersey), NASBA, (Organon Teknika, Durham, North Carolina) or SDA, Becton Dickinson, (Franklin Lakes, New Jersey), assays in which peptide nucleic acids recognize and bind target sequences and assays in which reagents recognize mismatches between the specimen's nucleic acid sequence and a known standard. These are variations of modifying or making multiple copies of specific nucleic acid templates for the purpose of detection, and when performed directly on an immobilized biological specimen are within the scope of either this invention or the heretofore mentioned U.S. Patent Numbers 5,382,511 and 5,451,500.

Microporous matrices are generally used in the field of molecular biology as a solid support media for proteins or nucleic acids transferred from electrophoretic gels. The Southern transfer technique is used to blot nucleic acids from gels onto a matrix in order to detect specific sequences by hybridization. In other instances, nucleic acids are filtered or spotted directly onto the matrix. The mechanism of the binding of proteins or DNA to matrices depends mainly on a combination of hydrophobic interactions, hydrogen bonding,

and salt bridges. Nitrocellulose matrices were the first type of media used for Southern transfer and required baking in a vacuum oven to make the bond formed between the medium and the nucleic acid essentially permanent, permitting stringent assay conditions and reuse of the blot after removal of a bound probe. One criteria for solid supports is that all reactive sites on the surface surrounding the bound molecule be eliminated or rendered inert to non-specific interactions. Properties of different types of matrices made of diazotized media, nylon and ion exchange matrices are matched to different applications and the size of the molecules to be retained. DEAE anion exchange matrices are used for reversible transfer-elution to isolate RNA or DNA wherein different buffers favor the binding or release of the biomolecules to the medium.

More hydrophilic matrices have been formulated for better wetting and less flow resistance. Manufacturers have developed blood separation media to trap white blood cells preferentially. Another fibrous medium separates the cellular and noncellular fractions of whole blood, as it wicks through the porous spaces in the matrix. The chromatographic effect that occurs during wetting with a given volume of whole blood displays a constant ratio of 25% for the cell-free plasma fraction in the Hemadyne matrix, Pall Corporation, Port Washington, New York. The pore structure of the matrix and its low binding affinity allows even high molecular weight proteins to migrate unimpeded and separate in an aqueous phase apart from the blood cells. These matrices present a quick way to either filter white cells from blood or separate biomolecules in the plasma away from the red blood cells. The manufacturer recommends extracting the nucleic acids from the matrix by using a proteinase enzyme to free the DNA from the immobilized white blood cells. The cell lysate, or a more purified nucleic acid portion, is then added to a

polymerase chain reaction (PCR). Separating white cells or biomolecules away from the red cells is useful in sample preparation because the large amount of hemoglobin in blood is known to poison enzymatic reactions such as PCR.

SUMMARY OF THE INVENTION

The method and device of this invention minimizes the quantity of a specimen required for a diagnostic result because the nucleic acids present in an immobilized specimen are labeled or amplified directly without the losses that are associated with extracting them from the specimen. The present invention immobilizes a small amount of biological or clinical specimen such as blood, needle biopsy aspirate, cell smear, cell print or ultra-thin tissue specimen onto a supporting matrix material, and dehydrates the specimen quickly to prevent degradation of nucleic acids. The matrix material containing the immobilized specimen is then rehydrated with either a labeling or amplifying reaction mixture for the detection of nucleic acids sequences of diagnostic interest.

The present invention is useful in reducing the quantity of reagents needed for processing the specimen. Fibrous materials increase the surface area relative to volume over solid supports. Reagents fill the void volume of a fibrous matrix and readily access the target nucleic acids expected to be present in an immobilized biological sample. Sample preparation is therefore miniaturized and simplified by processing the sample immobilized on a fibrous matrix. The matrix material to which the sample is dried may be rinsed or pretreated to remove any interfering components of the specimen and dehydrated a second time before detection.

The present invention improves the containment and integrity of patient specimens by collecting and transporting the specimen via the matrix material. The specimen is dried onto the solid-support matrix and amplified without using any of the standard methods to remove nucleic acids from cells or viral particles. The immobilized specimen, and the nucleic acids present, are treated less harshly than standard treatments with organic solvents. There is no need to recover and transfer nucleic acids from one reaction vessel or mixture to another. More specifically, the present invention enables automated processing by integrating the sample preparation with an enzymatic labeling or amplification step.

In accordance with the present invention, the preferred embodiment of the device comprises a fibrous matrix that is of a thin, flat shape rather than spherical or cuboidal, wherein a biological specimen is brought into contact with the total surface area of the fibrous matrix and then dehydrated by evaporation. A preferred embodiment of the size and density of matrix fibers is dependent upon the type of specimen.

In yet another embodiment, the invention herein relates to a device comprising a fibrous matrix attached to a handle for the purpose of touching the matrix to biological tissue. The device collects a cellular specimen more easily than trying to hold the tissue specimen and to touch it to a matrix or glass slide because the smaller matrix surface can be directed to a specific area of the specimen.

In a broad aspect, the component device of the invention comprises means for immobilizing a biological specimen by first drying it to a matrix support and then filling the void volume of the matrix with liquid reagents to amplify, label or hybridize specific

nucleic acids for detection. The invention is readily adaptable for use in an automated analyzer for DNA diagnostic tests.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A and 1B illustrate genetic products amplified from human blood immobilized and dried on different matrix materials and which are visualized after electrophoresis in agarose gels and showing bands the size of the expected targets.

FIGURE 2A and 2B illustrate genetic products of viral amplification from immobilized cell culture supernatant, whole blood, and plasma which was fractionated from whole blood by lateral flow through Hemadyne matrix material before immobilization, and visualized after electrophoresis in agarose gels.

FIGURE 3A and 3B illustrate agarose gel electrophoresis of products, amplified from equine dermal cells that were lifted from glass and plastic culture dishes by contact with a matrix material, immobilized and processed on the matrix material.

FIGURE 4 illustrates agarose gel electrophoresis products amplified from human cheek cells collected, immobilized and processed on a matrix material.

FIGURE 5 illustrates agarose gel electrophoresis products amplified from serial dilutions of whole blood immobilized on a matrix from as few as 100 white cells.

FIGURE 6 illustrates agarose gel electrophoresis products amplified from mRNA, as well as DNA, present in human cells immobilized and processed on a matrix material.

FIGURE 7 is a drawing of an embodiment of a sample collecting device for the analysis of nucleic acids.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention broadly comprises a method to immobilize biological and cellular specimens on a matrix for detection of target nucleic acids in the specimens. In accordance with the present invention, the preferred embodiment of the device comprises a matrix that is of a thin, flat shape rather than spherical or cuboidal, wherein a biological specimen is brought into contact with the surface area of the matrix and then dehydrated by evaporation. Dehydration is more rapid if the matrix is thin. Dehydrating is significant because the volume of the original sample may be larger, relative to the reaction volume, than if the sample was introduced as a liquid into the same reaction volume. In other words, dehydration is a way to concentrate the cells or virions containing nucleic acids from an aqueous, liquid biological sample and process more nucleic acid analytes in a smaller reaction volume and in a smaller space. A membranous matrix comprising more thread-like fibers and smaller fiber diameters increases the surface area of binding sites to immobilize all of the different components of a biological specimen. Additionally, a surface area with more binding sites than needed to adhere just the cells also serves to bind and effectively sequester those molecules which may inhibit nucleic acid modifying and amplifying enzymes.

The capillary air spaces in a fibrous matrix causes liquids and soft tissue cells to flow and spread throughout the matrix. This wicking or absorbing property of fibrous matrix materials causes more cells to adhere to a fibrous matrix having an outer dimension equivalent to an area of a smooth, flat glass slide. Different biological tissues vary

considerably in cell density and in the viscosity of their intercellular matrix material. A fluid tissue like whole blood is expected to wick further into the void spaces of a matrix than a tissue such as adenoids that are less fluid. A matrix comprised of a low density of fibers will contain a larger specimen volume than a high density matrix with a lesser void volume. The physical dimensions and composition of the matrix are selected on the basis of the general size and viscosity of the specimen so that the density of its fibers is sufficient to absorb the liquid volume of the specimen and immobilize the cellular components.

Individual cell clusters, or cells comprising desired characteristics, may be identified visually or microscopically and collected by physical contact with a fibrous matrix. The matrix containing the selected cellular specimen may be dried and analyzed for specific DNA and RNA content without isolating nucleic acids from the cellular material. A 3 x 3 millimeter square area may immobilize upwards of 200,000 cells from a biological specimen; however the sample may only need to contain ten to twenty cells or gene targets for amplification in order to increase the number of target copies to a detectable level. The capacity of a fibrous matrix to immobilize cells, is a desirable feature to incorporate into a device for analyzing genetic targets.

The number of cells needed in a diagnostic sample is determined statistically based on whether the target nucleic acid sequences occur commonly or rarely in a cell population. A fibrous matrix having one-eighth inch sides (3.2 millimeters) and a depth of 0.020 to 0.040 inch (0.5 - 1 millimeter) that is filled with a fluid layer has a volume equivalent to 5 - 10 microliters less the space filled by the fibers. Reducing the total reaction volume to a range of 5-25 microliters will conserve reagents and lower costs.

In cases where nearly all the cells in a specimen are expected to contain the target sequence, only a few cells are needed in order to amplify genetic sequences present even at only the single copy level per cell. The specimen surface area needed for a few cells is in the range of less than 100 square microns and the volume of reagents is in the range of a few picoliters. In cases where the target is expected to be a rare event, the specimen surface area may be accordingly increased in order to contain around 25 microliters of reagents for thousands of cells. Liquid droplets of cells dry onto a planar surface with cells affixed generally in a monolayer, if the cells are not too concentrated in the solution. Embedded or frozen tissue is routinely cut into 4-10 microns sections that are nearly a monolayer or at most a few cells thick. With the device of the invention, cells are collected and immobilized similarly in a monolayer-like arrangement, but by means that do not involve embedding in a liquid media phase that solidifies for microtome sectioning. The immobilized, dehydrated specimen preserves labile mRNA and is directly accessible for the diffusion of amplifying and hybridizing reagents.

The dimensions of small fibrous matrix pieces, and a device for handling and enclosing them in a reaction chamber, will depend upon the density of cells and other biomolecules in the specimen. It is expected that specimens containing more cells and biomolecules will occupy more surface area of the matrix, depending on their ability to flow. The reaction volume needs to provide sufficient reactants so as to not limit biochemical activity. The surface area of the fibrous matrix used should not be in large excess of what is needed to immobilize the specimen since the remaining reactive sites on the matrix may bind the amplification enzymes, primers and nucleotides, labeled or not, limiting their availability for the amplification or detection reaction.

Chambers, as described in applicant's U. S. Pat. No. 5,346,672, and having a specimen area capacity of a 15 millimeter-diameter circle on a microscope slide are routinely used in our laboratory to prevent evaporation during thermocycling slide specimens. As a matter of standard procedure, we removed the reaction liquid after amplification and analyzed it by agarose gel electrophoresis because products from the slide specimen were found to be present in the solution. Results were used to confirm the size of products amplified from the specimen immobilized on the slide. Frequently, the slide specimen area will contain 50,000 cells or less and cover only a 3 millimeter diameter area on the slide surface. The chamber contains a liquid volume of approximately 50 to 75 microliter. Under conditions when the cells are covering only a portion of the positively-charged surface of an adherent slide enclosed in the reaction chamber, it is necessary to include in the amplification mix 0.1-0.01% bovine serum albumin (BSA), or a similar blocking agent, in order to bind the unoccupied plus charges on the slide and block the polymerase enzyme and nucleic acid molecules from binding to the slide. The amount of BSA needed for an optimum amplification reaction may be empirically determined. Using 0.01% BSA in immobilized sample amplifications has become routine as a precaution to cover reactive sites on matrix materials that may interfere with enzyme activity.

The applicants performed hundreds of genetic detection reactions on biological specimens, namely blood and cheek cells. Fresh whole blood from fingerpricks and cheek cells collected by either whole mouth fluids, transmucosal exudate and buccal smears, were immobilized on a dozen of different matrix materials from several manufacturers (namely, Gelman Sciences, Ann Arbor, MI; Magna Separations Inc. (MSI), Westboro, MA; Pall BioSupport, Port Washington, NY; Porex Technologies, Fairburn, GA;

Schleicher & Schuell, Keene, NH; Tetko, Briarcliff Manor, NY; 3M, St. Paul, MN). It was convenient in the laboratory to detect by amplifying and subjecting the product and controls to agarose gel electrophoresis and UV illumination of ethidium bromide fluorescence. However, it is understood there are many other ways known in molecular biology to detect nucleic acids, many of which detect fewer molecules than can be visualized by the detection method used herein.

EXAMPLE I

Early experiments in our laboratory confirmed that ISA products from whole blood spotted on matrices were the same as the products obtained from amplifying purified human DNA spotted on the same type of matrix. Bands in Figure 1A demonstrate the same specific 524 base-pair fragment, amplified by using primer extensions from two opposing primers complementary to a region of the human p53 gene. 100 µl reactions were comprised of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% BSA, 0.2 mM of each of the 4 standard dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers and 1.25 Units of Taq DNA polymerase. Thermocycling conditions were an initial denaturation at 94 °C for 4 min., followed by 30 cycles of 94 °C, 45"; 52 °C, 45"; 72 °C, 1' and then a final extension at 72 °C for 5'. Following thermocycling, twenty microliters of each reaction were electrophoresed on a 1.5% agarose gel and products visualized with UV-illumination of the ethidium bromide stained gel. Lanes 2 and 3, represent ISA product from five-microliter, dried whole blood spots on Hemadyne, rinsed briefly with water and 1 mM HCl, respectively, and lane 4, product form a dried spot of 100 nanograms of human genomic DNA. (Lane 1 is Boehringer Mannheim DNA molecular Marker VI).

Reports by others in the literature that the presence of hemoglobin inhibits enzymatic amplification apparently is minimized when the blood is immobilized on a matrix. Further experiments compared immobilizing blood on a variety of different matrix materials. Fresh whole human blood was collected directly from a finger prick and 2 µl was spotted onto each of several small squares of different matrix materials. The sizes of the matrices were approximately 4-9 mm^2 . Within seconds the blood flowed into the intramatrix spaces and in a few minutes the blood components dried to the surface of the matrix fiber material. For the inactivation of blood borne pathogens, the matrix materials containing blood are heated at 82 °C for 15 min. Each matrix was washed twice for 5 min each with 100 µl of water and redried at 82 °C for 15 min. The matrices containing the cells were then amplified in thin-walled tubes under the following conditions in 50 µl reactions: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% BSA, 0.2 mM of each of the 4 standard dNTPs, 2.25 mM $MgCl_2$, 0.5 µM primers and 1.25 Units of Taq DNA polymerase. Thermocycling conditions were an initial denaturation at 94 °C for 3'50", followed by 30 cycles of 94 °C, 45"; 60 °C, 45"; 72 °C, 1' and then a final extension at 72 °C for 5'. Following thermocycling, twenty microliters of each reaction were electrophoresed on a 3% agarose gel and products visualized with UV-illumination of the ethidium bromide stained gel. Primers, used for identity testing and recognizing a single copy locus (locus 34), generated the appropriate 115 base-pair amplification product from blood samples immobilized on the following matrices as shown in Figure 1B: lane 2, Nylon 3-3710 , (Tetko); lane 3, Nitex Nylon (Tetko); lane 4, Magna nylon (MSI); lane 5, Hemadyne (Pall); lane 6, Leukosorb (Pall); lane 8, polyester twill (Tetko); lane 9, PeCap polyester 17/9 (Tetko); lane 10, PeCap polyester 43/29

(Tetko); lane 11, PeCap polyester 150/41 (Tetko); lane 12, PeCap polyester 210/35 (Tetko); lane 13, Tetex polyester (Tetko). Lane 1 represents a control reaction containing 100 ng purified human genomic DNA and Lane 14 is Boehringer Mannheim DNA molecular Marker XIII. There was no band apparent in lane 7, representing NFWA matrix (Gelman). The invention is a method for the analysis of nucleic acids of a biological specimen, comprising (a) immobilizing a given specimen on the surfaces of a fibrous matrix material, (b) amplifying a targeted sequence region of said specimen's nucleic acid, and (c) detecting the presence or absence of said targeted sequence of said specimen's nucleic acid. Accordingly, the invention is a method for the analysis of nucleic acids wherein the biological specimen is blood.

EXAMPLE 2

Hemadyne lateral flow membrane was used to separate whole blood into a cellular fraction and a cell-free plasma fraction in order to investigate the capability of the RNA retrovirus, Equine Infectious Anemia Virus (EIAV) to flow through the membrane with a plasma fraction and to be identified by ISA. One microliter of the supernatant of EIAV-infected equine dermal cell culture, was spotted onto Hemadyne alone or mixed with 5 microliters of human whole blood first and then spotted on Hemadyne, as well as whole blood alone. Sections of the Hemadyne membranes containing either the dried culture supernatant or plasma, which had been separated from the cellular fraction, were excised and used for ISA. The matrix pieces were added to separate reverse transcription reactions using SuperScriptII Reverse Transcriptase (Life Technologies, Gaithersburg,

MD) according to manufacturer's instructions and incorporating an EIAV-specific primer. One-fourth of each reverse transcription reaction was amplified by Taq polymerase with an EIAV primer pair. In Figure 2A, bands representing product amplified from EIAV virus immobilized on the Hemadyne were visualized in all lanes except lanes 5 and 6 in which no EIAV had been added to the blood; lane 2, unwashed, dried culture supernatant spot; lanes 3 and 4, washed, dried culture supernatant spot; lanes 7 and 8, EAIV in the plasma fraction. The invention is a method of detecting viral nucleic acids present in a whole blood specimen, comprising (a) applying said whole blood specimen to a lateral flow matrix; (b) separating said whole blood specimen into a cellular fraction on a first portion of said lateral flow matrix and a plasma fraction on a second portion of said lateral flow matrix; (c) drying and immobilizing said plasma fraction on a second portion of said lateral flow matrix;(d) saturating said plasma fraction on a second portion of said lateral flow matrix with amplification reagents to enzymatically amplify viral nucleic acid targets present in said plasma fraction; (d) determining whether said plasma fraction of said whole blood specimen contains said viral nucleic acid targets.

In other experiments, results shown Figure 2B, 10 microliters of horse blood, with EIAV-containing supernatant added (lanes 6-9), and without EIAV (lanes 2-5), was applied to one end of a Hemadyne strip and flowed laterally across the strip. After lateral flow had ceased, the Hemadyne strip was dried and then cut into four sections and each section was amplified using rTth DNA polymerase (Perkin Elmer, Foster City, CA), EIAV primers and appropriate buffers and thermocycling conditions. The blood that was pipetted onto a portion of the matrix strip is represented in lanes 2 and 6; and flowed laterally through portions of the matrix represented in lanes 3-4 and 7-8, respectively, and

portions of the matrix containing plasma where the flow ended are represented in lanes 5 and 9. The bands in Lanes 6-9 are evidence that viral particles are deposited over the entire area of the matrix. The blood was taken from a horse previously infected with EIAV and expected to have very low amounts of virus remaining. The bands in lanes 6-9 and a faint band in Lane 1 (visible on the original photograph) are evidence that ISA is capable of detecting virus in blood when either sufficient virus present, the volume of blood sample is adequate or a method more sensitive than an agarose gel is used to detect amplified product. When the virus was present at very low copy number, as in the infected horse blood, a band was seen after amplification only in the portion of the matrix where blood was deposited directly on the membrane; when excess virus was added to the blood, the virus was detectable in all portions of the lateral flow matrix. In experiments performed at the veterinary school from where the horse's blood was obtained, extensive measures are required to amplify detectable EIAV from this same horse by standard means. First white cells are isolated by a Ficoll gradient, nucleic acids are purified from the white cells, and two sequential PCR are performed with a portion of the first reaction mix being used in the second reaction. Viral RNA or DNA is therefore available more readily for enzymatic amplification from whole blood specimens and component fractions of whole blood by the ISA method than standard PCR methods.

The invention is a process of detecting viral nucleic acids present in whole blood, comprising (a) applying a specimen of whole blood to a fibrous matrix; (b) drying said specimen of whole blood on the surfaces of said fibrous matrix; (c) applying reagents to said fibrous matrix to enzymatically amplify viral nucleic acid targets that may be present in said specimen of whole blood; (d) determining whether said whole blood specimen

contains said viral nucleic acid targets. Accordingly, by comparing results to known quantities of virus and blood volumes, the relative quantities of said viral nucleic acid targets present in said specimen may be measured. A standardized amount of a mimic virus, one constructed to have a sequence variation to make it detectable, as well as complementary sequence structure so that it would be amplified in the same manner, may be added to and immobilized together with the specimen in order establish a positive control for the reaction and quantify relative amounts of virus particle present in the specimen. The invention is a method of detecting viral nucleic acids further comprising means to determine quantities of said viral nucleic acid targets present in said specimen.

EXAMPLE 3

Evidence for the ability of a fibrous matrix material to actively pick up and immobilize cells for detection of a gene sequence present at one or two copies per cell is shown in Figure 3. Equine dermal cells that had been grown to a confluent monolayer on either plastic tissue culture plates, or on sterile glass chamber slides, were washed twice with phosphate buffered saline and drained briefly to remove excess PBS while also keeping the cells adhered to the plastic or glass. Small squares of matrix (approximately 4-9 mm^2) were gently touched to the surface of a portion of the moist cell monolayer to collect cells. The matrices were then transferred to reaction vessels and dried and washed as described above. Reaction mixtures were as described for Figure 1B, except for a change to 1 mM $MgCl_2$ and primers recognizing single-copy, equine gene sequences for tumor necrosis factor and a 55 °C

annealing temperature were used. (Lane 1 contains Boehringer Mannheim molecular weight marker XIII.) The expected 245 base-pair amplification product was obtained in Figure 3, lanes 2 and 3 from horse genomic DNA and also from cells grown on either plastic (lanes 4-9) or glass (lanes 10-15) and printed onto one of three matrices for immobilized sample amplification. (Nylon 3-3710, lanes 4,5,10,11; PeCap polyester 17/9: lanes 6,7,12,13; PeCap polyester 43/29: lanes 8,9,14,15).

Small pieces of matrices are difficult to handle whether manufacturing or using them as devices. Squares cut from Nylon 3-3710 were adhered with a double-sided adhesive transfer tape to a polycarbonate film of the same length and width of the matrix square, but having a thickness of 0.007 inch (178 microns), to support the matrix and make it more easily handled. The results of Figure 3B, lanes 5 and 7 show that the matrix's ability to pick up cells and amplify nucleic acids from them, under conditions as described in Figure 3A, was not altered or compromised when a piece of the matrix was attached to an adhesive and backing for support. (Figure 3B, lanes 2 and 3 contained products amplified from horse genomic DNA and lanes 4 and 6 contain products on unsupported pieces of matrix, touched to cells on plastic and glass culture dishes, respectively, as in Figure 3A.) The sandwiched device comprising a matrix, adhesive and backing is in communication with enzymatic amplification and hybridization reagents; therefore all materials used in fabricating different embodiments of the invention would be tested for biocompatibility. The applicants have had experience with many different materials with which to fabricate devices and have identified some that do and some that do not affect ISA for the detection of nucleic acids. The invention is a device for analyzing the genetic sequence of small

amounts of the nucleic acids of a specimen, comprising (a) a fibrous matrix, (b) means to support said fibrous matrix, (c) means to contact said fibrous matrix with a biological specimen, (d) means for cellular material of said biological specimen to attach to said fibrous matrix, and (e) means to detect the presence or absence of specific genetic sequences in the nucleic acids of said cellular material transferred to said fibrous matrix from said biological specimen.

EXAMPLE 4

In another experiment, a piece of matrix was attached to the end of a handle with an adhesive that had been shown previously to be compatible with ISA. The unit was autoclaved and used to collect cheek cells directly from the mouth of a volunteer. After collection, each matrix with adhesive backing was detached from the handle and transferred to reaction vessels, dried and processed as described for Figure 1B. The matrices were rehydrated with a reaction mix containing the components and products were visualized as described previously for Figure 1B. In Figure 4, primers recognizing a single copy, identity-testing locus (locus 60) generated a 169 base-pair amplification product both in control reactions containing human genomic DNA target (lanes 2 and 3) and in ISA reactions containing cheek tissue cells immobilized on Nylon 3710, (lanes 5 and 6). No product was evident in reactions containing no target (lane 4). (Lane 1 contains Boehringer Mannheim DNA molecular weight marker XIII.) The invention is a device for analyzing the genetic sequence of small amounts of the nucleic acids of a human subject, comprising (a) a fibrous matrix, (b)

means to support said fibrous matrix, (c) means to contact said fibrous matrix with a human subject to obtain a biological specimen, (d) means for cellular material of said biological specimen of said human subject to attach to said fibrous matrix, and (e) means to detect the presence or absence of specific genetic sequences in the nucleic acids of said cellular material transferred to said fibrous matrix from said biological specimen of said human subject.

EXAMPLE 5

Biological specimens were diluted to derive the sensitivity level, or copy number, at which a particular nucleic acid sequence could be detected. The number of cells containing nucleic acids in human blood is relatively constant so blood was diluted and each dilution was analyzed for the detection of a genetic sequence represented with two alleles per cell. Fresh human blood was collected from two individuals by fingerprick and either spotted directly onto a matrix or diluted in phosphate buffered saline and then spotted directly onto a matrix. Each dried matrix was added to a reaction vessel with a polymerase chain reaction mix containing the components described for Figure 1B, with a substitution of 1.5 mM $MgCl_2$. Reactions were thermocycled using the same primers described for Figure 1B. In Figure 5, lanes 2 and 3 demonstrate the 115 base-pair product generated by specific amplification of human genomic DNA. The product amplified by ISA from 2 µl of immobilized human blood can be seen in lanes 4 and 9. Two microliters of serial dilutions (1:5, lanes 5

and 10; 1:10, lanes 6 and 11; 1:50, lanes 7 and 12; 1:100, lanes 8 and 13) are also sufficient to generate a visible product on the original photograph of the gel. (Lane 1 contains Boehringer Mannheim DNA molecular weight marker XIII.) The results of Figure 5 demonstrate that a single-copy human gene can be amplified from 0.02 μl of immobilized human blood, or approximately 100 cells. Bands were visible in other experiments from dilutions estimated to have as few as 20 immobilized cells.

Because immobilized sample amplification does not depend upon isolation techniques in which the recovery of nucleic acids may vary from sample to sample, rapid analysis of either single cells or a few cells immobilized on a matrix is more achievable. In certain research applications it may be desirable to analyze the nucleic acids of a single cell, but the interpretation of results for diagnostic purposes will be more reliable when based on more than a single cell. An advantage of ISA is the capability to analyze the genetic material of a small amount of biological specimen for a variety of different reasons: surgical biopsy material may be sparse as in needle aspirates of suspected breast tumors and lymph nodes; a small region may be selected to determine the extent or border of the malignant characteristics of a tumor by analyzing somatic genetic mutations; and regions of tissue may be analyzed after genetic therapy to determine the spatial or temporal distribution of the genetic intervention. Anatomical orifices and surface lesions are non-surgical sources of biological specimens available for ISA. For example, skin lesions caused by ticks are a more direct source of material to test for nucleic acid evidence of parasites than peripheral blood. Fresh cervical tissue from suspicious zones may be transferred by

touching to a matrix for the analysis of abnormal cytology, thereby eliminating scraping or incisions to do so.

In cases in which a few cells or viruses are contained in a larger volume of biological fluid than can conveniently be applied and dried to a small matrix, for example in the spinal fluid from lumbar punctures of patients with meningitis, the matrix itself may be used to capture the cells or viruses. Matrices have traditionally been used for separation and filtration by using the sieving mechanism of their pore size to separates biomolecules based on size. For ISA the capture does not need to be based on sieving but rather a natural affinity of cellular and viral surface biomolecules to attach to the matrix material. In our laboratory two different matrix materials were layered, each capable of binding cells, but the cells adhered to the matrix material it first contacted. The liquids continued to wick into the capillary spaces of the second matrix even though few cells were carried with it. It is obvious that for the purpose of keeping the matrix material small enough for a low volume reaction and minimizing the surface area of the matrix which enters the reaction, that the collecting device may be backed with an absorbent material which is not part of the matrix.

In many instances, fluids undergoing filtration are pressurized to force fluids through the matrix voids. Without pressurization, the capillary force of the aqueous liquids is the chief mechanism in filling the matrix voids, initially replacing the air in these spaces. Either using pressure, or only the capillary wicking action of the matrix, as a separation system is compatible with ISA. The fibrous and chemical nature of the matrices that comprise the sample collection device, and their ability to bind and immobilize the biological specimen containing genetic material to its surface area for the

analysis of nucleic acids, is enabling for the device of the invention. The device of the invention may optionally contain absorbent material in addition to the primary matrix.

EXAMPLE 6

The analysis of RNA expression from immobilized cells and RNA viruses is within the scope of the invention. In Figure 6 reverse transcription and amplification of messenger RNA was demonstrated in human cells immobilized on a matrix. A derivative of human lymphoblastoid CEM cells growing in suspension culture were concentrated by centrifugation and then resuspended in culture media containing serum. Aliquots containing approximately 10,000 cells were applied to small squares (4-9 mm^2) of matrix, dried and washed as described above. The combined reverse transcriptase and amplification system sold by Boehringer Mannheim, as their Titan RT-PCR System, containing a 1x reaction buffer of 1.5 mM $MgCl_2$ and DMSO, 0.2 mM dNTP's, 0.01% bovine serum albumin, 5 mM dithiothreitol, 0.5 µM of each primer and 1 µl of Titan enzyme mix was added directly to the dried matrices. Reactions were subjected to an initial 30 minute incubation at 50 °C to allow reverse transcription of mRNA, followed by 30 cycles of 94 °C, 45"; 60 °C, 45"; 72 °C, 15" and a final extension incubation at 72 °C for 5 min. Oligonucleotide primers homologous to two exons of the tumor necrosis factor gene generate a 568 base-pair product of DNA amplification and a 266 base-pair product resulting from RT-PCR amplification of the spliced tumor necrosis factor messenger RNA, lanes 2 and 3 in Figure 6. The template for amplification in Lane 2 are purified nucleic acids isolated

from human CEM cells and in lane 3, the CEM cells immobilized on Nylon 3-3710 are amplified by ISA. In methods for amplifying RNA, other than the ISA method, strong denaturing solutions are added to cell lysates to preserve RNA. Extensive measures are taken to recover RNA rapidly before it is degraded. In other ISA experiments (data not shown), heating the immobilized cells and matrices in water for 20 minutes at 90 ° C. eliminated evidence of RNA amplification, but the capability to amplify DNA was unchanged. One advantage of ISA for mRNA detection is a simple means to detect mRNA directly from cells of interest without extraordinary measures.

The invention is a method for the analysis of a small amount of ribonucleic acid of a biological specimen, comprising: (a) immobilizing a biological specimen on surfaces of a fibrous matrix material; (b) amplifying a targeted region of said biological specimen's nucleic acid sequence; (c) means to detect the amplified targeted region of said biological specimen's nucleic acid sequence; in order to identify the presence or absence of at least one particular ribonucleic acid sequence in said biological specimen.

One embodiment of the invention device to collect and immobilize biological specimens is shown in Figure 7. The device 10 is comprised of a matrix 12 for the purpose of immobilizing a biological specimen and a handle 14 to facilitate placement of the matrix 12. The handle 14 is provided to manipulate a small matrix 12 into close proximity of the desired area of the biological specimen and to contact the specimen area and transfer cells 17 from the biological specimen to the matrix 12. The device 10 will further have means to separate the handle 14 from the matrix 12 in a manner that controls placement of the matrix 12 during detachment, allowing the matrix 12 to be placed in a reaction vessel. Quality control manufacturing of the matrix 12 is

expected to maintain consistency in fiber diameter, chemical composition and density. Depending upon the nature of the fibrous matrix, cutting may weaken the ability of the matrix fibers to stay together and require special cutting methods and a backing 16 for physical support. Fabrication of devices 10 comprising very small pieces of matrix material may be facilitated by affixing a length of matrix material to other supporting materials before cutting it into individual pieces for each device.

The embodiment of the device 10 in the drawing of Figure 7 illustrates a design that enables a large assembly of devices 10 to be made at one time. First, a long narrow strip, circa 6 millimeter-width, of matrix material 12 is affixed by means of an adhesive 18 to a plastic backing 16 of the same size to form the matrix sandwich 20.

Handles 14 for a set of devices are fabricated by folding and creasing a sheet of plastic as long as the matrix sandwich 20. A row of openings 22 is made in the sheet so that each handle 14 will have one opening 22. A second adhesive 24 is applied to the first side of the plastic sheet before folding and the matrix sandwich 20 is placed on the second side of the plastic sheet with the backing 16 facing the openings 22. The adhesive 24 fastens the backing 16 of the matrix sandwich 20 to the second side of the plastic sheet through the openings 22. The sheet is then creased at least twice on either side of the row of openings and folded by bringing opposite ends, parallel to the row of openings, and pressing the first side together with the adhesive 24 inside to keep the plastic sheet folded. The matrix sandwich 20 remains affixed to the second side of the plastic sheet by contact with the adhesive 24 through the openings 22. When the assembled sheet is cut perpendicular to the row of openings,

between each pair of openings 20, the matrix strip will also be cut into small pieces in a way that each device has the 12 matrix facing outward as drawn in the single device 10 of Figure 7.

The devices 10 are packaged for individual use and opened at the site of sample collection where a technician will remove the device by its handle 14, guide and contact the matrix 12 to the biological specimen area. To detach the matrix sandwich 20 and place it in a reaction vessel, the handle 14 is unfolded by peeling the first sides apart and away from the adhesive 24, releasing the matrix backing 16 from the adhesive 24 in the area of the opening 22. Adhesives 18 and 24 may be either liquid materials spread over the matrix backing 16 and plastic sheet forming the handles 14, respectively, or a linered, pressure sensitive adhesive transfer tape.

Yet another embodiment of the device comprises a narrow roll of matrix, with or without a backing, contained in a housing having means to advance the roll, cut and release sections of matrix as needed. Housings for applying labels to products and tape to packages are examples of similar mechanisms. Another embodiment of a housing means is similar to a pill dispensor, in which individual matrices are stacked in a cylinder with a first means to expose one matrix at a time for gentle pressure to contact the specimen and a second means to eject the matrix from the container. The invention is a device according to Example 3, further comprising a handle or dispensing apparatus.

It is understood that other embodiments of a sample collecting and processing device are within the scope of this invention, regardless of the mode of manufacturing or the intended type of biological specimen. A preferred embodiment of the device of

the invention are shown as a matrix sandwich comprising nylon and polyester fibers, supported by a plastic backing and a handle from which the matrix may be detached. Different embodiments of a matrix material, upon which the sample is immobilized, include, and are not limited to, fibrous matrices, comprising one or more types of natural or synthetic fibers, that are fibers laid down in a pattern to encourage lateral or vertical flow, absorbent wicking materials, a woven matrix, two or more matrices layered together and the like. The matrix may comprise a sticky surface or a pattern made by manufacturing techniques to increase surface area of a material to which cells and viral particles naturally adhere, or have been coated to increase adhesion. The matrix itself may be an internal part of a reaction vessel. A handle is useful to manipulate and collect a biological specimen, but optional because it is not necessary. The sample collecting matrix, and any other supporting material that becomes part of the reaction, are selected on the basis that they do not inhibit ISA or the analysis of nucleic acids.

A further property of the sample collection device is that it may provide a measure of the amount of biological sample that is collected. For example, a measured amount of a particular fibrous matrix in the device will absorb a predetermined quantity of a liquid biological specimen. Another example of using the device to measure the biological sample is that when the size and adherence of collecting surface area that is provided with the device has been established to collect the same amount of a particular biological specimen within an acceptable range, the results may be expressed in quantifiable terms. Therefore, the invention may be a

method of detecting and determining the relative quantities of nucleic acid targets, such as the prevalence of viral particles, present in a given quantity of specimen.

It is possible to determine the amount of clinical specimen needed for immobilization that will routinely amplify fewer genetic targets than other methods. Immobilizing the sample eliminates extraction procedures and therefore none of the target nucleic acids originally present in the sample are lost due to the fact they are not recovered during the extraction steps. An aqueous solution of biochemically active enzymes and amplification reagents is readily absorbed into a dehydrated matrix and rehydrates the biological specimen . The rehydrated specimen immobilized on the matrix is subjected to appropriate temperature conditions and amplified products in the reaction solution are detected by any number of means known in the art.

In order to detect multiple mutations from the same sample, it is possible to use a high density probe array representing different sequence combinations to which the complementary nucleic acids of the amplified product bind upon diffusing from the immobilized cells. By immobilizing a clinical specimen on a first matrix surface and detection probes on a second surface, both in the same reaction chamber, sample preparation can be integrated with amplification and detection into a single reaction chamber. There are numerous schemes for labeling, transmitting and detecting signals based on the hybridization of complementary nucleic acid sequences that may be applied to detect products of immobilized sample amplification, eliminating the need for gel electrophoresis altogether. The more sensitive the detection, the less amplification product is needed to transmit a signal. The invention is further a method for analyzing the genetic sequence of small amounts of the nucleic acids of a specimen, comprising the

following steps: (a) drying and immobilizing said nucleic acids of said specimen on a first internal surface of a reaction chamber; (b) immobilizing nucleic acids in a known array on a second surface of the reaction chamber; c) amplifying or labeling said targeted regions of said nucleic acids of said specimen; (d) means for said labeled or amplified nucleic acids of said specimen to bind said nucleic acids immobilized in said known array; (e) and means of detecting the presence, absence or amount of said targeted regions of said nucleic acids of said specimen by interpreting to what genetic sequence of said known array, said labeled or amplified nucleic acids of said specimen are bound by their sequence complementarity.

Applicant (Inventors): Marilyn J. Stapl Rebecca Sundseth Attorne ocket
Serial or Patent No.: -- No: STM05011
Filed or Issued: --

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[X] the owner of the small business concern identified below:

[ ] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Gene Tec Corporation
ADDRESS OF CONCERN 205 Winterberry Ridge Drive
Durham, NC 27713

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Immobilizing and Processing Specimens on Matrix Materials for the Identification of Nucleic Acid Sequences by inventor(s) Marilyn J. Stapleton and Rebecca Sundseth described in

[X] the specification filed herewith
[ ] application serial no. _____, filed _____
[ ] patent no. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a non profit organization under 37 CFR 1.9(e). *Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME None
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date of which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and after having been warned that willful false statements, and the like, so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF PERSON SIGNING MARILYN J. STAPLETON
TITLE OF PERSON OTHER THAN OWNER _____
ADDRESS OF PERSON SIGNING
205 Winterberry Ridge Drive, Durham

SIGNATURE Marilyn J. Stapleton DATE April 14, 1997

Stapleton & Sundseth

Sheet 1 of 10

FIG. 1A

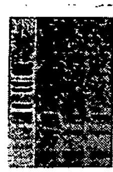

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FIG. 1B

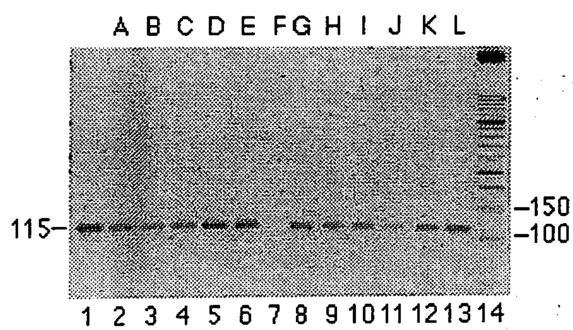


FIG. 2A

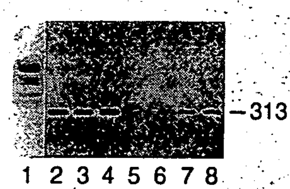


FIG. 2B

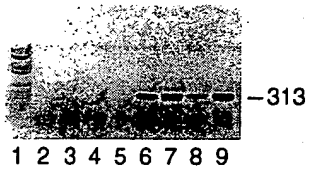
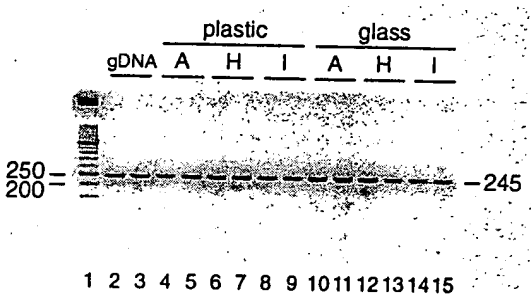


FIG. 3A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200
245
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FIG. 3B

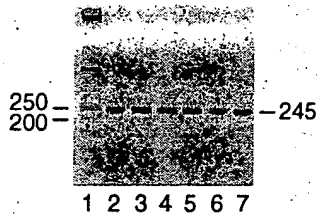

FIG. 4

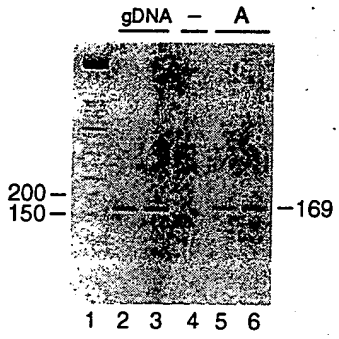

FIG. 5

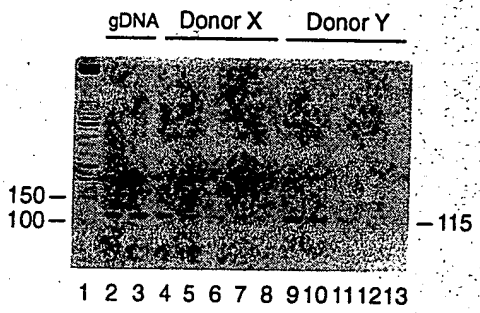

FIG. 6

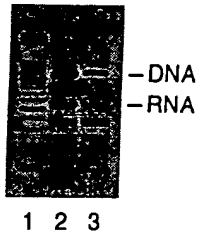

FIG. 7